



Artigo / Article

Simplified flow cytometric assay to detect minimal residual disease in childhood with acute lymphoblastic leukemia

Detecção de doença residual mínima em crianças com leucemia linfoblástica aguda por citometria de fluxo

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The detection of minimal residual disease (MRD) is an important prognostic factor in childhood acute lymphoblastic leukemia (ALL) providing crucial information on the response to treatment and risk of relapse. However, the high cost of these techniques restricts their use in countries with limited resources. Thus, we prospectively studied the use of flow cytometry (FC) with a simplified 3-color assay and a limited antibody panel to detect MRD in the bone marrow (BM) and peripheral blood (PB) of children with ALL. BM and PB samples from 40 children with ALL were analyzed on days (d) 14 and 28 during induction and in weeks 24-30 of maintenance therapy. Detectable MRD was defined as $\geq 0.01\%$ cells expressing the aberrant immunophenotype as characterized at diagnosis among total events in the sample. A total of 87% of the patients had an aberrant immunophenotype at diagnosis. On d14, 56% of the BM and 43% of the PB samples had detectable MRD. On d28, this decreased to 45% and 31%, respectively. The percentage of cells with the aberrant phenotype was similar in both BM and PB in T-ALL but about 10 times higher in the BM of patients with B-cell-precursor ALL. Moreover, MRD was detected in the BM of patients in complete morphological remission (44% on d14 and 39% on d28). MRD was not significantly associated to gender, age, initial white blood cell count or cell lineage. This FC assay is feasible, affordable and readily applicable to detect MRD in centers with limited resources. *Rev. bras. hematol. hemoter.* 2008;**30**(4):281-286.

Key words: Minimal residual disease; acute lymphoblastic leukemia; flow cytometry; children; peripheral blood.

Introduction

Early treatment response is an independent prognostic factor in childhood acute lymphoblastic leukemia (ALL) and exceedingly important to confirm the patient's risk group and

to define treatment intensity.^{1,2} The rate of clearance of leukemic cells from the peripheral blood (PB) and bone marrow (BM) can be measured by morphologic criteria, e.g., the number of circulating lymphoblasts after one week of chemotherapy,^{3,4} or the number of blasts in the BM during or

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at the end of induction therapy.^{5,6} Despite clinical usefulness, measurements of residual leukemia by morphologic analysis can be insensitive and imprecise. Patients who achieve complete morphological remission may still have a high tumor burden and, therefore, a high risk of relapse. Minimal residual disease (MRD), as assessed by more sensitive and precise methods, has emerged as a powerful prognostic indicator in childhood ALL and has recently been incorporated in many treatment protocols to identify the risk of relapse and to define therapeutic strategies.⁷⁻¹²

Several techniques have been developed over the past 10 to 15 years that go beyond morphology in assessing response to treatment. In ALL, the most reliable methods for MRD detection are polymerase chain reaction (PCR), to detect fusion gene transcripts,¹³ rearranged immunoglobulin (Ig) and T-cell receptor genes,^{14,15} and the detection of aberrant immunophenotypes by flow cytometry (FC).^{8,9,16,17} Both techniques can detect one leukemic cell or more in every 10,000 normal BM or PB cells. Choosing a specific technique depends on the desired sensitivity and the applicability of the technique to accomplish each specific clinical objective.^{12,18} When optimally performed, molecular studies are more sensitive, and may require fewer tumor cells in the sample. FC is usually less expensive and faster than molecular methods.¹⁹⁻²¹

Leukemia-associated immunophenotypes, used to gauge MRD by FC, are usually absent in normal hematopoiesis. These aberrant phenotypes usually involve cross-lineage antigen expression (i.e., the expression of myeloid markers in lymphoid blasts), maturational asynchronous expression (co-expression of two or more antigens that are not simultaneous in normal differentiation), over- or under-expression, or ectopic antigen expression (the presence of a marker that would be normal if the cell was in another homing area).^{7,8,16,17}

One of the greatest obstacles to perform FC is the high initial cost and, in Brazil, the need to import all reagents. Nonetheless, at least in most centers, a single-laser flow cytometer is already available to diagnose childhood ALL. Thus, we conducted a prospective single center study to evaluate MRD in the BM and PB of children with ALL using a 3-color FC assay with a limited antibody panel that is reproducible in other centers and countries with limited resources.

Patients and Method

From March 2003 to October 2004, 40 children with newly diagnosed ALL admitted for treatment at our institution were eligible for the present study. Diagnosis was based on the criteria of morphological classification proposed by FAB and on immunophenotyping with monoclonal antibodies. Patients were from 0.4 to 16 years old (median 5 years) with 23 boys and 17 girls. Seven had T-lineage ALL (T-ALL) and 33 had

B-cell-precursor ALL (BCP-ALL). The children were treated according to the guidelines of the Brazilian Cooperative Group for the Treatment of Childhood Leukemia (GBTLI-LLA/99) protocol (n=30) or the ALL Berlin-Frankfurt-Munster (BFM)-95 protocol (n=10). This study was approved by the University Ethics Committee and informed consent was obtained from all parents or guardians.

BM and PB samples were obtained at diagnosis to identify the leukemia-associated immunophenotypes and during induction on days 14/15 (d14) and days 28/33 (d28) and in weeks 24 to 30 of maintenance therapy to evaluate MRD. Samples were collected in EDTA_{K3} and processed within 24 hours. MRD was determined in the BM and PB in 212 follow-up samples (112 BM and 100 PB). MRD studies were only performed in samples with more than 1,000 leukocytes/mm³. BM morphology was analysed at the same time points.

Immunophenotyping at diagnosis

Leukemic blasts were immunophenotyped using a two or three color combination of monoclonal antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCp) or CyChrome: IgG1-FITC/PE/CyChrome; CD1a-FITC; CD2-PE; CD3-PE/CyChrome; CD4-FITC; CD7-PE; CD8-PE; CD10-PE; CD15-FITC; CD19-FITC/CyChrome; CD20-FITC; CD33-FITC/PE; CD45-FITC; CD79a-PE (Becton Dickinson - Pharmingen, San Jose, CA); CD10-FITC; CD22-PE; CD34-FITC/PE; CD45-PerCp; MPO-FITC; terminal deoxynucleotidyl transferase (TdT)-FITC (Becton Dickinson Biosciences, San Jose, CA); CD13-PE; IgM-FITC (Dako, Carpinteria, CA). The stain and lyse/wash technique was used, as previously reported.²² Data acquisition was performed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) equipped with the Cell Quest computer program. A total of 15×10^3 non-gated events were acquired. At least one useful aberrant marker combination was identified at diagnosis and then used to monitor MRD in the follow-up samples.

MRD evaluation

Matched non-reactive fluorochrome-conjugated antibodies and the CD4-FITC/CD8-PE/CD3-CyChrome combination were used as negative and positive controls, respectively. A two-step acquisition procedure was applied. In the first step, a total of 15×10^3 non-gated events were acquired. In the second step, a live gate (LG) based on the lineage marker expression was set: CD19 for BCP-ALL and cytoplasmic (cy) CD3 for T-ALL, using low/ intermediate side scatter characteristics (SSC - lymphoid scatter). The total numbers of cells, usually 10^5 - 10^6 events passing through the flow cytometer were recorded for each LG acquisition.²²⁻²⁵

The expression of all markers was analyzed within CD19⁺ or cyCD3⁺ lymphocyte SSC gate to determine the presence of any cell subset with the aberrant marker.²²⁻²⁵ In patients with no aberrant marker, MRD was monitored by

triple-stainings: CD45/CD10/CD19; CD34/CD22/CD19; CD20/CD10/CD19.^{23,24} Data analysis was achieved with the Paint-a-Gate Pro software (Becton Dickinson, San Jose, CA). A cluster of at least 10-20 events with the patients' aberrant marker expression and adequate SSC was considered as MRD. "Detectable MRD" was defined as the expression of the leukemia-associated immunophenotype in 0.01% or more of the total events present in the sample.²²

Statistical analysis

Differences in the distribution of presented clinical and biological features according to the existence of MRD in the BM during induction were compared by Fisher's exact test. Cohen's kappa was used as a measurement of agreement between MRD in BM and PB. The Spearman's correlation coefficient (r_s) was determined to compare MRD levels in paired BM-PB samples. For this analysis, only paired BM-PB samples with detectable MRD in at least one sample were included. The *P*-values were always two-tailed and values < 0.05 were regarded as statistically significant. The Statistical Package for Social Science (SPSS) version 10.0 was used for statistical calculations.

Results

In this study, 35 of the 40 patients (87%) had leukemia-associated immunophenotypes suitable for MRD studies by FC (Table 1). Most patients with BCP-ALL had at least one aberrant marker (28/33 - 85%), with 20 (61%) having two or more. All seven patients with T-ALL had suitable phenotypes, based on the simultaneous detection of nuclear TdT and cyCD3. One T-ALL patient also had aberrant myeloid markers.

MRD results and association with clinical risk factors

About half of the patients had detectable MRD in the BM on d14 (20/36 - 56%) and on d28 (18/40 - 45%) but only 3% in weeks 24-30. At the end of induction (d28), MRD levels

Table 1. Leukemia-associated immunophenotypes identified at diagnosis

ALL	Aberrant immunophenotype	N	%
BCP- lineage (n=33)	CD13 co-expression	15	45
	CD45 under-expression	13	39
	CD10 over-expression	10	30
	CD33 co-expression	7	21
	CD10 over-expression and CD20 asynchronous expression	4*	21
	cyIgM and CD34 asynchronous expression	6	18
	CD34 over-expression	4	12
T-lineage	TdT and cyCD3 ectopic expression	7	100
(n=7)	CD13 co-expression	1	14

*Only 19 patients could be tested for the simultaneous CD10 (bright) and CD20 expression

Table 2. Patients' presenting features, according to the presence of MRD in BM during the remission induction therapy

Presenting feature	MRD (d14)			MRD (d28)		
	<0.01%	≥ 0.01%	P*	<0.01%	≥ 0.01%	P*
Sex						
Male	10 (62.5)	11 (55)	.741	14 (63.6)	9 (50)	.523
Female	6 (37.5)	9 (45)		8 (36.4)	9 (50)	
Age, y						
<1 ≥10	2 (12.5)	5 (25)	.426	4 (18.2)	6 (33.3)	.300
≥1<10	14 (87.5)	15 (75)		18 (81.8)	12 (66.7)	
WBC count, cells/mm ³						
<50 000	11 (68.7)	12 (60)	.731	14 (63.6)	10 (55.6)	.748
≥50 000	5 (31.3)	8 (40)		8 (36.4)	8 (44.4)	
Cell lineage						
B	16 (100)	15 (75)	.053	19 (86.4)	14 (77.8)	.613
T	0 (0)	5 (25)		3 (13.6)	4 (22.2)	

Values are numbers (%) of patients. *with Fisher's exact test

were 0.01-0.1% in 11 patients (27.5%), 0.1%-1% in 2 (5%) and ≥ 1% in 5 (12.5%). For the remaining 22 patients (55%), leukemic cells were below the detection threshold using this technique (<0.01%). The presence of detectable MRD (MRD⁺) in the BM during induction was not significantly associated to gender, age, white blood cell count or cell lineage in this limited number of patients (Table 2).

Correlation between MRD and BM morphology

Of the 112 follow-up BM samples studied by FC during ALL therapy, 13 (12%) had ≥ 5% blasts identifiable by morphology. In all of these samples, leukemic cells were also identified by FC varying from 0.8% to 78% of the BM cells (median, 25%). Among the 99 (88%) samples in complete morphological remission (< 5% blasts), 73 (74%) had no detectable cells expressing leukemia-associated immunophenotypes. However, 26/99 (26%), remained MRD⁺ ranging from 0.01-10.5% (median: 0.03%).

MRD in the BM vs. PB

Of the 112 follow-up BM samples, 100 had paired PB available for analysis. Twelve PB samples could not be analyzed due to leukopenia. Eight of the corresponding BM samples were MRD⁺ and four had no detectable MRD.

A total of 39 BM samples (35%) had more than 0.01% leukemic cells (0.01-78%; median 0.14%). Thirty-one matched PB samples were available: 23 (74%) had ≥ 0.01% circulating blasts, whereas eight (26%) had none (< 0.01%). In the 23 MRD⁺ PB samples, levels ranged from 0.01% to 32% (median 0.045%). No detectable MRD was observed in the remaining 69 BM-PB paired samples.

During induction, MRD measurements were similar in the BM and PB. Of the 28 patients with

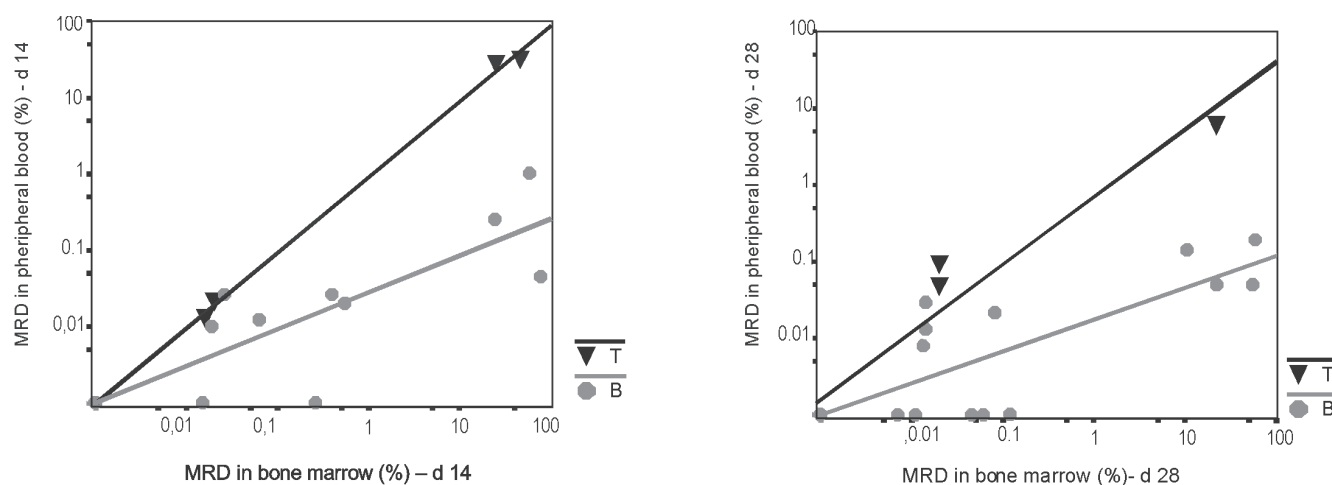


Figure 1. Distribution of MRD levels in BM and PB of patients with T-lineage ALL and B-lineage ALL during the remission induction therapy. Values represent the percentage of residual disease determined by flow cytometry in paired BM and PB samples. Symbols indicate T-lineage ALL (▼) and B-lineage ALL (●)

paired BM and PB samples on d14, half had detectable MRD. In two of these 28 patients (14%), MRD was detected only in the BM (kappa 0.857, $P < 0.001$). On d28, at the end of the induction, 36 patients were evaluated and 16 (44%) had positive samples. Of these, five were positive only for the BM (kappa 0.710, $P < 0.001$). Only one patient had detectable MRD in the BM in weeks 24-30.

T-ALL patients had similar levels of MRD+ in the BM and in the PB at all time points. The Spearman's correlation coefficient (r_s) was 1.0 ($P < 0.05$) on d14 and 0.984 ($P < 0.001$) on d28. Therefore, in T-ALL, the PB reflected the BM MRD while in BCP-ALL, MRD was more prevalent and present at higher levels in the BM than in the PB (Figure 1). The Spearman's correlation of PB and BM MRD was strong in BCP-ALL on d14 ($r_s = 0.892$; $P < 0.001$) and on d28 ($r_s = 0.776$, $P < 0.001$), although the MRD levels in the PB were usually lower than in the paired BM samples.

Discussion

The morphologic detection of leukemic cells during therapy is an important prognostic factor. However, it is difficult to distinguish leukemic blasts from early lymphocytes and undifferentiated normal hematopoietic cells only by morphology. FC has the potential of overcoming these limitations.⁷⁻⁹ In our study we showed that 44% (d14) and 39% (d28) of patients with morphologically normal BM during induction actually had measurable MRD.

Sequential monitoring of MRD during the first months of therapy provides information on the response to treatment and has already been proved to be a powerful and independent indicator of outcome in childhood ALL.^{22,26-32} Nevertheless, the use of MRD monitoring can be limited by the cost of these techniques.

In our study, we used a relatively low-cost FC method,

based on a limited panel of antibodies that was applicable in 87% of patients. This frequency of aberrant immunophenotypes is similar to what has been reported in other series.^{8,9,17,33-37}

The FC method described in this study is also affordable, rapid and easily performed in any cancer center equipped with a basic single-laser flow cytometer, the FC-equipment available in most Brazilian institutions. All these features may facilitate the prospective validation of this method in treatment protocols in developing countries.

Our study corroborates the theory that variations in the occurrence of immature lymphoid precursors, which resemble the phenotype of leukemic cells, may influence MRD detection.^{26,34,38,39} Regenerative time points (weeks 24-30) were characterized by a limited sensitivity for MRD assessment in a high proportion of BCP-ALL cases due to the preponderance of resurgent normal very immature precursor B-cells (data not shown).

Kerst *et al.*²¹ reported that neither high percentages nor the virtual absence of immature B-cells appeared to influence flow cytometric estimates of MRD. In spite of this, we found that options for investigating MRD may not depend on leukemia itself, but also on time point-related factors such as total count and developmental stage of normal cells. These observations were also suggested by Dworzak *et al.*²⁶ Hence, strategic placement of assessments into non-regenerative phases of treatment (when the numbers of immature B-cells are too low or absent) may allow a more accurate determination of MRD using few marker combinations that are sufficiently broad to assess most patients as was proposed in a study recently published by Coustan-Smith *et al.*⁴⁰

For many years the material of choice to monitor MRD in ALL has been BM. Repeated BM aspirates cause discomfort and are a traumatic experience for children. Therefore, PB is being used for MRD testing.⁴¹⁻⁴⁵

We found differences in the PB MRD of patients with T and BCP-ALL. In T-ALL, cells expressing leukemia-associated immunophenotypes were consistently present in the PB and BM at remarkably similar levels. These findings and those of other studies suggest that the PB can be used for MRD investigation in T-ALL.^{44,45} However, in BCP-ALL, some patients had no detectable cells with leukemia-associated immunophenotypes in the PB despite their presence in the BM. In addition, BM samples had higher MRD levels than the PB. This result is consistent with other PB MRD studies.^{41,42,44,45} In the study by Coustan-Smith *et al.*,⁴⁴ the presence of MRD in the PB of patients with BCP-ALL denoted a more aggressive leukemia with an extremely high risk of recurrence. Therefore, PB MRD assays may also be clinically useful in BCP-ALL.

In conclusion, FC is a fast, applicable and relatively inexpensive technique to detect MRD in children with ALL. The method we herein describe must be prospectively validated in the context of the treatment protocols used in developing countries.

Resumo

A detecção de doença residual mínima (DRM) é um importante fator prognóstico na leucemia linfóide aguda (LLA) infantil e fornece informações sobre a resposta ao tratamento e o risco de recaída. Entretanto, os altos custos das técnicas utilizadas limitam seu uso nos países em desenvolvimento. Desta forma, realizamos um estudo prospectivo para avaliar a citometria de fluxo (CF), utilizando três fluorescências e um painel limitado de anticorpos monoclonais, como método de detecção de DRM em medula óssea (MO) e sangue periférico (SP) de crianças com LLA. Amostras de MO e SP de 40 crianças portadoras de LLA foram analisadas nos dias (d)14 e d28 da indução e nas semanas 24-30 da terapia de manutenção. Foram consideradas como DRM+ as amostras que apresentaram $\geq 0,01\%$ das células com o fenótipo aberrante (FA). Oitenta e sete por cento dos pacientes apresentaram FA ao diagnóstico. No d14, 56% das amostras de MO e 43% do SP apresentaram DRM. No d28, foi detectada DRM em 45% e 31% das amostras de MO e SP, respectivamente. A porcentagem de DRM na MO foi similar à do SP nos casos de LLA-T, mas aproximadamente dez vezes maior na LLA de precursor-B. Foi detectada DRM na MO de 44% e 39% dos pacientes que estavam remissão morfológica nos d14 e d28, respectivamente. Não foi demonstrada associação significativa entre a presença de DRM e sexo, idade, leucometria inicial e linhagem celular. Esta técnica de detecção de DRM por CF é relativamente barata e pode ser aplicada em centros com recursos limitados. Rev. bras. hematol. hemoter. 2008;30(4):281-286.

Palavras-chave: Doença residual mínima; leucemia linfóide aguda; citometria de fluxo; criança; sangue periférico.

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